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The molecular, functional and phylogenetic characterization of PGE_2 receptors reveals their different roles in the immune response of the teleost fish gilthead seabream (*Sparus aurata* L.)

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ABSTRACT

Prostaglandin E_2 (PGE_2) plays an important role in immune activities in teleost fish, including seabream. However, receptors involved in PGE_2 signaling, as well as the pathways activated downstream, are largely unknown. In this study, one ortholog of mammalian PTGER1, PTGER3 and PTGER4, and two of PTGER2 (Ptger2a and Ptger2b) were identified and characterized in gilthead seabream. *In silico* analysis showed that all these receptors possessed the organization domain of G protein-coupled receptors, with the exception of Ptger2b. The corresponding *in vivo* studies revealed that they were expressed in all the tissues examined, the highest mRNA levels of *ptger1* and *ptger3* being observed in the spleen and of *ptger2a* and *ptger4* in the blood. Bacterial infection induced higher mRNA levels of *ptger2a, ptger3* and *ptger4* in peritoneal exudate (the site of bacterial injection). In addition, head kidney acidophilic granulocytes and macrophages displayed different *ptger1, ptger2a, ptger3* and *ptger4* expression profiles. Furthermore, in macrophages the expression of the receptors was weakly affected by stimulation with bacterial DNA or with PGE_2, while in acidophilic granulocytes stimulation resulted in the upregulation of *ptger2a* and *ptger4*. Taken together, these results suggest different roles for seabream PGE_2 receptors in the regulation of the immune responses.

1. Introduction

Prostanoids including PGE_2 are bioactive lipid-derived autacoids (Ricciotti and FitzGerald, 2011) involved in a broad array of physiological and pathological processes (Gamil et al., 2015; Kobayashi and Narumiya, 2002). They are derived from the arachidonic acid released from cell membranes (Calder and Grimble, 2002) in response to a variety of stimuli (Harris et al., 2002; MacKenzie et al., 2010; Ricciotti and FitzGerald, 2011; Sargent et al., 1999), which is subsequently converted into to PGH_2 through the action of two rate-limiting enzymes: constitutive (COX-1) and inducible (COX-2) cyclooxygenases (Wang and Dubois, 2010). PGH_2, is converted into several prostanoids by the action of the respective synthases (Narumiya et al., 1999).

The divergent effects of PGE_2 have been involved in a large spectrum of biological processes (Hao and Breyer, 2008; Ning et al., 2014; Miller, 2006), including both pro- and anti-inflammatory mechanisms, ranging from lower vertebrates (fishes) (MacKenzie et al., 2010; Gómez-Abellán and Sepulcre, 2016; Tyrkalska et al., 2016) to human (Calder and Grimble, 2002; Hsu et al., 2014; Fattahi and Mirshafiey, 2014; Miller, 2006).

In mammals, the pleiotropic effects of PGE_2 are mediated by binding to one or to a combination of four receptors subtypes PTGER1-PTGER4 (also named EP1-EP4) (Harris et al., 2002; Qu et al., 2015), belonging to the family of rhodopsin-like seven transmembrane-spanning, G-protein-coupled receptors (Guo et al., 2015). These receptors can be grouped into three groups of prostanoid receptors according to the corresponding associated G-protein subunit, and hence to the intracellular second messengers and the effect triggered by them (Yokoyama et al., 2013). The group of relaxant receptors include EP2 and EP4,

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Abbreviations

AGs	acidophilic granulocytes
COX	cyclooxygenase
cAMP	cyclic adenosine monophosphate
C	cysteine
dbcAMP	dibutyryl-cAMP
D	aspartic acid
EP	E prostaglandin receptor
EDTA	ethylenediamine tetraacetic acid
EMBOSS	European molecular biology open software suite
EMBL	European molecular biology laboratory
EBI	European bioinformatics institute
EST	Expressed sequence tag
ENA	European nucleotide archive
EL	extracellular loop
FCS	fetal calf serum
GPCR	G protein–coupled receptor
GTP	guanosine triphosphate
G	arginine
HK	head kidney
HEK	human embryonic kidney cells
IgG	Immunoglobulin G
IL	intracellular loop
LPS	Lipopolysaccharide
MACS	magnetic-activated cell sorting
NCBI	national center for biotechnology information
NF-κB	nuclear factor kappa B
ORF	open reading frame
PGE_2	prostaglandin E2
PE	peritoneal exudates
PAMPs	pathogen associated molecular patterns
PG	prostaglandin
PTGER	prostaglandin E receptor
PGH_2	prostaglandin H_2
PKA	protein kinase A
RT-qPCR	quantitative RT-PCR
ROS	reactive oxygen species
R	arginine
S	serine
S.E,	standard error
TMD	transmembrane domain
T	threonine
UTR	untranslated region
VaDNA	*Vibrio anguillarum* genomic DNA

Table 1
Primer sequences used for cloning and qRT-PCR.

Gene	Forward/Reverse	Primer sequence (5'->3')	Technique
ptger1	Forward	TATCGGAGAGCACCTGTCCA	qRT-PCR
	Reverse	CTGGCACTTAGCGATGAGGT	
	Forward	AAAAAGGATCCATGTTAGCGTTGAGCCACT	cloning
	Reverse	AAAAAGCGGCCGCAACGTGGTTGACATCATTCT	
ptger2a	Forward	TTCTTCAGCCTTGCGACCAT	qRT-PCR
	Reverse	TGCACCAAATCCCGCAAAAG	
	Forward	AAAAAGGATCCATGGGGAATCCAGAGAAC	cloning
	Reverse	AAAAAGCGGCCGCTGGTAATAGAGTTTCTGCAG	
ptger2b	Forward	AAAAAGGATCCATGGGGAATCCAGAGAAC	cloning
	Reverse	AAAAAGCGGCCGCGAAGTCAACGGTCAACACTT	
ptger3	Forward	TCCCTCACCAGATCCTGGAC	qRT-PCR
	Reverse	AGTCTGGGTCTCCTTCCGAT	
	Forward	AAAAAGGATCCATGACTATGGACGCTTCTGA	cloning
	Reverse	AAAAAGCGGCCGCGCTACTTTGTGGTTTATGGATGT	
ptger4	Forward	GGGCAGAGATCCAGATGGTG	qRT-PCR
	Reverse	TGTGCAGCTGATTCGCAAAG	
	Forward	AAAAAGGATCCATGAACTCCACGGTAAAAC	cloning
	Reverse	AAAAAGCGGCCGCTATGCATTTCTCTTGTATGTTTGC	
il8	Forward	GTTTTTGAAGAGGGCTGAG	qRT-PCR
	Reverse	TTTGCTTGAAGTTTCACTGG	

Table 2
Genbank or Ensembl accession numbers of Ptger used in this study.

Specie	Ptger1	Ptger2a	Ptger3	Ptger4
Homo sapiens	AAH51286	AAD44177	AAI18660	AAI13524
Mus musculus	NP_038669	NP_032990	NP_035326	NP_001129551
Gallus gallus	Unknown	ABM92941	ABF13295	NP_001074972 XP_424770
Xenopus tropicalis	XP_002935465	XP_002936949	XP_002936132	NP_001120554
***Danio rerio* H.**	ACX47465.1	zEP2a: NM_200635 zEP2b:NM_001030250 zEP2L: ABB88955.1	XP_009300798	NM_001039629
***Sparus aurata* L.**	XM_030412901.1	Ptger2a: XM_030410287.1 Ptger2b XM_030410288.1-	XM_030434233.1	XM_030436477.1
***Salmo salar* L.**	XP_014048970.1	XP_014000607.1	XP_014072350.1	NP_001167426.1
Larimichthys crocea	XP_010732951	XP_010742830	XP_010727602	KKF22113
Etheostoma spectabile	XP_032394488.1	XP_032362064.1	XP_032382424.1	XP_032395955.1

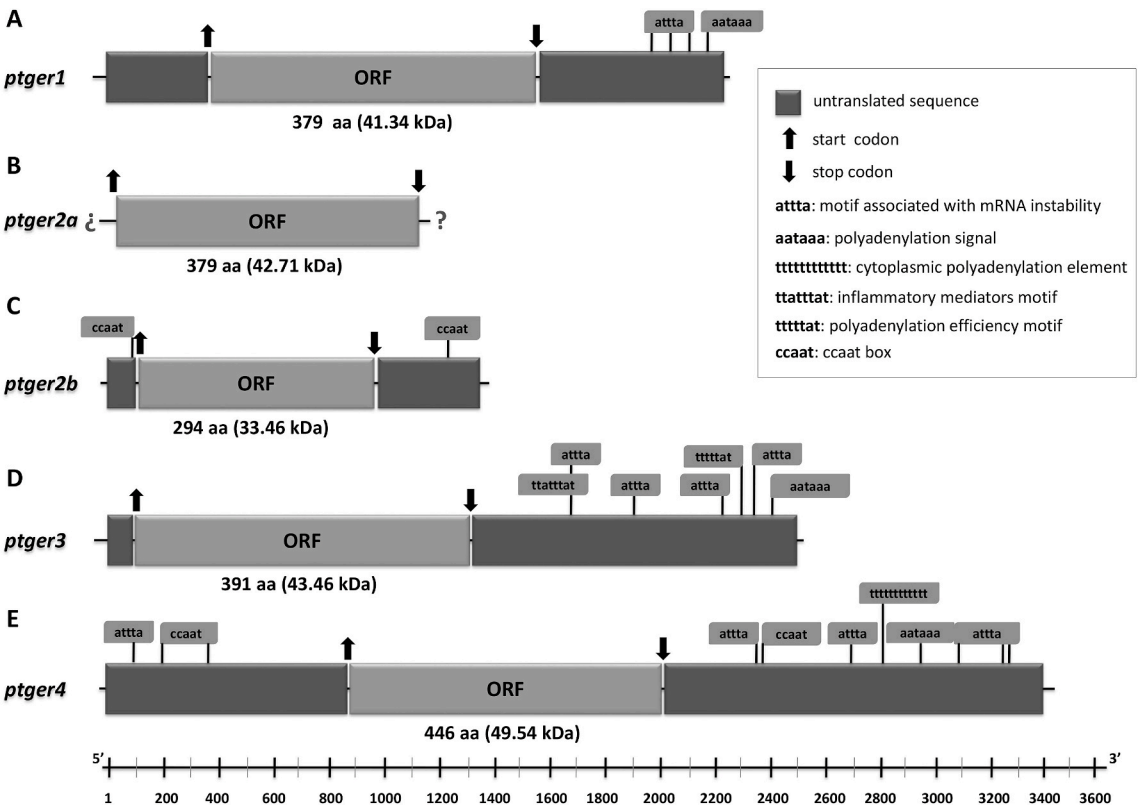


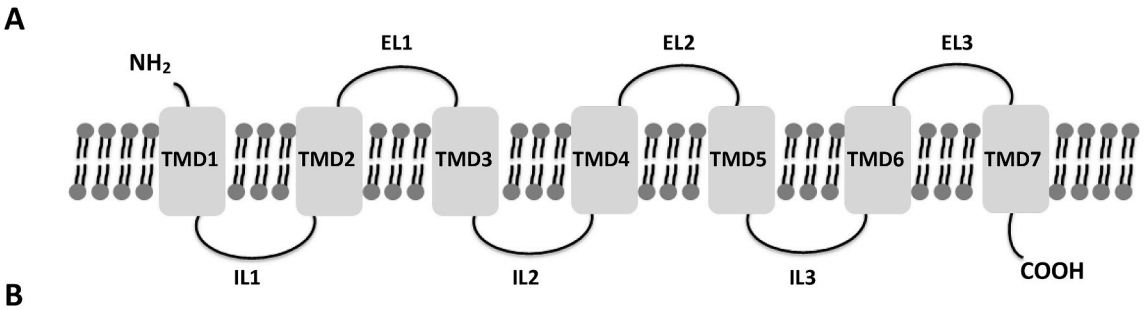
Fig. 1. Schematic representation of seabream Ptger subtype sequences. Schematic representation of seabream *ptger1* (A), *ptger2a* (B), *ptger2b* (C), *ptger3* (D) and *ptger4* (E).The open reading frames are represented in light gray boxes and the 5′- and 3′-UTR in dark gray boxes. The number of amino acids, as well as the molecular weight of the deduced amino acid sequences, are shown below each ORF. Also shown are the start (ATG, upward-pointing black arrow) and stop codon (TAA or TGA, downward-pointing black arrow) and motifs: **attta (**motif associated with mRNA instability), **aataaa** (polyadenylation signal), **ttatttat** (inflammatory mediator motif), **tttttat**(polyadenylation efficiency motif), **ttttttttttt** (cytoplasmic polyadenylation element) and **ccaat** (ccaat box).

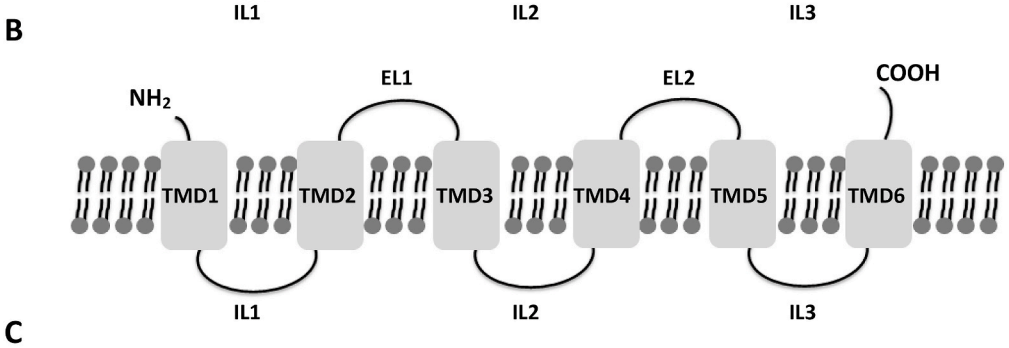
whose activation results in increased cAMP/PKA through $G\alpha_s$ and adenyl cyclase (Fujino et al., 2002), leading to smooth muscle relaxation. EP1 is coupled to $G\alpha_q$, which increases Ca^{2+} levels following the activation of phospholipase C (Fujino and Regan, 2005) and belongs to the group of contractile receptors. EP3 is generally coupled to Gi, leading to a decrease in cAMP via $G\alpha_i$ and resulting in the inhibition of muscle contraction (Narumiya et al., 1999), although different isoforms resulting from alternative splicing in the EP3 gene are coupled to different types of G protein, such as Gαs, Gαi and Gα12/13 (Tang et al., 2017).

Mammalian EP1-EP4 receptors exhibit distinctive levels of ligand-binding affinity, different selective expression, tissue distribution and subcellular localization (Sugimoto and Narumiya, 2007; Yokoyama et al., 2013). These receptors have been characterized at genomic (Locker and Buzard, 1990; Smock et al., 1999; Suganami et al., 2016; Yokoyama et al., 2013), proteomic (Margan et al., 2012; Stillman et al., 1999; Sugimoto and Narumiya, 2007; Yokoyama et al., 2013) and expression levels, both *in vitro* and *in vivo*, in response to a variety of stimuli (Birrell et al., 2015; Sakamoto et al., 2004; St-Jacques and Ma, 2011; Sheppe et al., 2018). Mouse EP3 and EP4 are the most widely PGE2 receptor distributed (Yokoyama et al., 2013) and both have a much higher affinity than EP1 and EP2 for PGE_2 (Fujino et al., 2002).

In teleost fish, the production of PGE_2 by immune cells has been described in a broad range of species, including black seabream (*Spondyliosoma cantharus*), black rockfish (*Sebastes melanops*), red seabream (*Pagrus major*), Atlantic cod (*Gadus morhua*), yellow croaker (*Larimichthys polyactis*), rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*) and gilthead seabream (*Sparus aurata* L.) (Gómez-Abellán and Sepulcre, 2016). Furthermore, there is evidence concerning the immunoregulatory activity of PGE_2 in fish: for example, the modulation of cytokine expression, ROS production and phagocytic responses (Gómez-Abellán and Sepulcre, 2016). More specifically, we have shown that PGE_2 leads to M2 polarization of macrophages in gilthead seabream (Montero et al., 2016), in a similar way to that seen in mammals (Harris et al., 2002). PGE_2 deactivates acidophilic granulocytes (AGs, the functionally equivalent cell to mammalian neutrophils) through the modulation of the cytokine expression profile (Montero et al., 2016). However, studies concerning the molecular characterization of PGE_2 receptors and their role in the immune response in teleost fish are scant. Mammalian homologs of EP1-4 have been identified and characterized in several fish species: zebrafish (*Danio rerio* H.) (Kwok et al., 2012; Tsuge et al., 2013), Atlantic salmon (*Salmo salar* L.) (Guo et al., 2015), *Bostrichthys sinensis* (Lai et al., 2014) and ayu (*Plecoglossus altivelis*) (Rong et al., 2016), EP4 being the most characterized (Fujimori et al., 2011; Gamil et al., 2015; Guo et al., 2015; Tsuge et al., 2013). They are involved in several functions such as reproduction (Fujimori et al., 2011, 2012), metabolic regulation (Busby et al., 2002) and the immune response (Guo et al., 2015; Rong et al., 2016). Ptger4 has also been considered as an early marker of T cells precursor in zebrafish development (Villablanca et al., 2007). However, our knowledge of the role of PGE_2 receptors in the teleost immune response is still limited, which makes a comparative analysis with their higher vertebrate counterparts quite difficult.

In previous studies, we showed that the cell-permeable analog of cAMP (dbcAMP) mimicked the effect of PGE_2 in the regulation of cytokine mRNA levels in stimulated macrophages but not in AGs. The variety of effects that PGE_2 elicits in both phagocytic cell types could reflect the presence of specific receptors in those cells and suggest that PGE_2 signals through EP2 and/or EP4 in seabream macrophages but not in AGs. Taking into consideration all the above, the aim of this study was





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                                                  TMD1                 IL1
Ptger2a      MGNPENDTCHYKQWIDPDKTPTISAIMFAAGIIGNVAALVILEVRRCRDSRNGGGRRRSL    60
Ptger2b      MGNPENDTCHYKQWIDPDKTPTISAIMFAAGIIGNVAALVILEVRRCRDSRNGGGRRRSL    60
               ************************************************************
                        TMD2                EL1                 TMD3
Ptger2a      FHILITSLVVTDLTGTCLISPLVQLSYHQNKTLTEMTHSKSVCRYFGLSMTFFSLATMAL    120
Ptger2b      FHILITSLVVTDLTGTCLISPLVQLSYHQNKTLTEMTHSKSVCRYFGLSMTFFSLATMAL    120
               ************************************************************
                              IL2                 TMD4
Ptger2a      LLTTALERCLAIGYPYFYNRHVTKKCAYITIPVVFLLCTLFCLLPFAGFGAYVQYCPGTW    180
Ptger2b      LLTTALERCLAIGYPYFYNRHVTKKCAYITIPVVFLLCTLFCLLPFAGFGAYVQYCPGTW    180
               ************************************************************
                     EL2                TMD5                 IL3
Ptger2a      CFIDMNPKETVHRVYANLYASVMLLLVLAIVSCNGFVVYQLYKMYQRRRRNGGSVISTKR    240
Ptger2b      CFIDMNPKETVHRVYANLYASVMLLLVLAIVSCNGFVVYQLYKMYQRRRRNGGSVISTKR    240
               ************************************************************
                                   TMD6                 EL3
Ptger2a      SHNERRSTSMAEEVEHLILLVFMTIIFIICTLPLVFRVYINSLWTNVESHRLDLIALRFI    300
Ptger2b      SHNERRSTSMAEEVEHLILLVFMTIIFIICTLPLVVRWQQNTHMNALKVLTVD-----F-    294
               ********************************.*   *:  . ::   :*     *
                        TMD7
Ptger2a      SVNSIIDPWVFIILSPSVLHFCWSSVCRAPVGITRGSVFKSSLARENSPANLELACPSLD    360
Ptger2b      ------------------------------------------------------------    294


Ptger2a      SNGQFQTAQTLPTAETLLP       379
Ptger2b      ------------------        294
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Fig. 2. Schematic domain organization and sequence alignment of seabream Ptger2a and Ptger2b. Diagrams showing the domain organization of seabream Ptger2a, which is also representative of Ptger1, Ptger3 and Ptger4 (A), and Ptger2b (B). TMD: transmembrane domain, IL: intracellular loop, EL: extracellular loop. (C) Alignment of seabream Ptger2a and Ptger2b sequences. (*) Identity in one position; () conservative substitutions in one position; (.) semiconservative substitutions in one position.

the identification and in silico characterization of gilthead seabream PGE_2 receptors (Ptger1-4) and to gain further insight into their role in the resolution of inflammation *in vivo* an *in vitro*. In particular, their distribution *in vivo* and modulation after bacterial challenge and their contribution to phagocytic cell functions were analyzed. These data pave the way for analysis of the role of PGE2 receptors during the immune response of teleost fish.

2. Materials and methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata*, Actinoperygii, Sparidae) were bred and kept at the Oceanographic Center of Murcia (Spain) in a 14 m^3 running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the

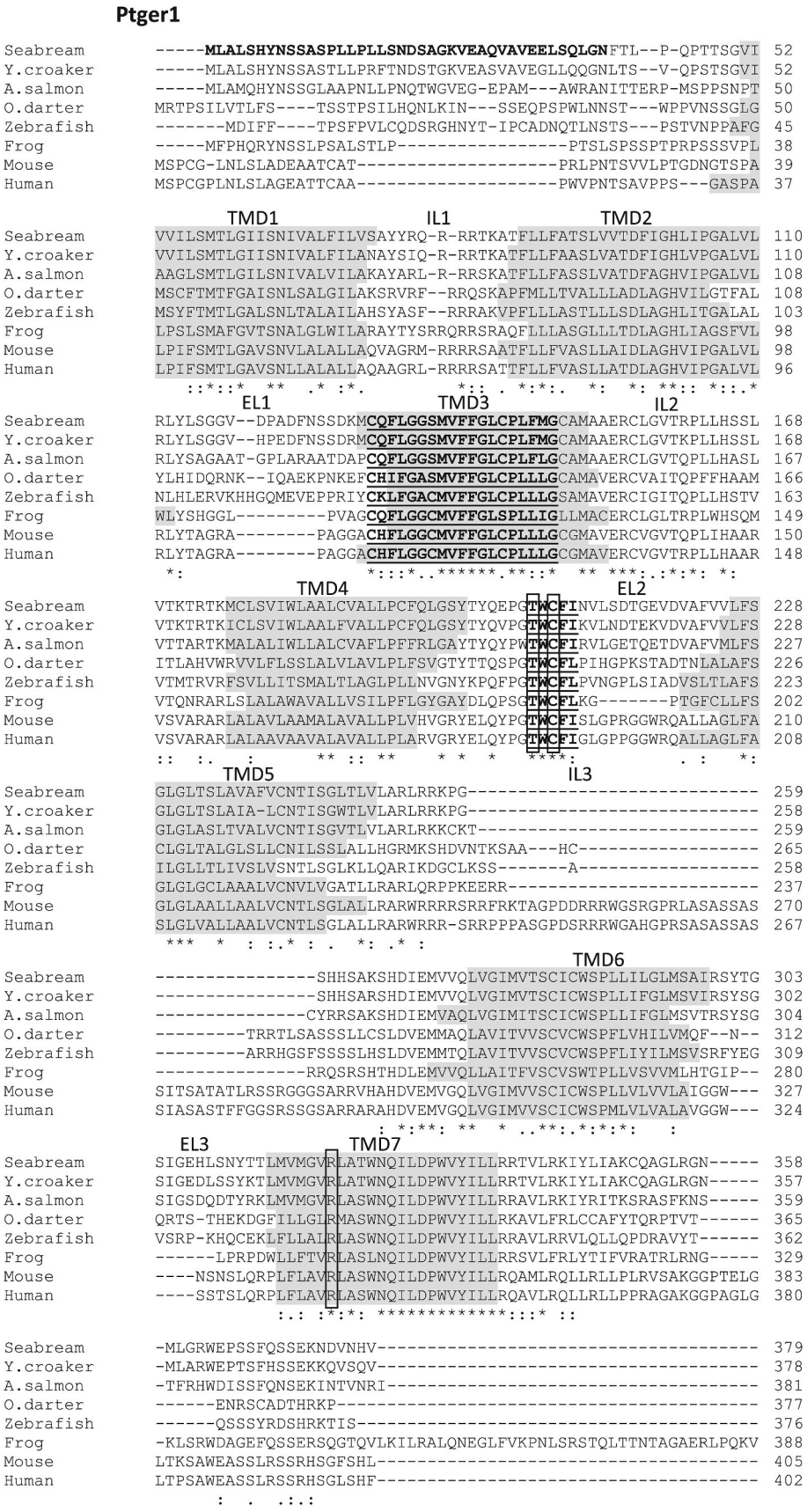

Fig. 3. Sequence alignment and domain organization of seabream Ptger1 sequence with known vertebrate Ptger1 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; () conservative substitutions in one position; (.) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in Table 2. Conserved motifs are in bold and underlined. Signal peptides are in bold.

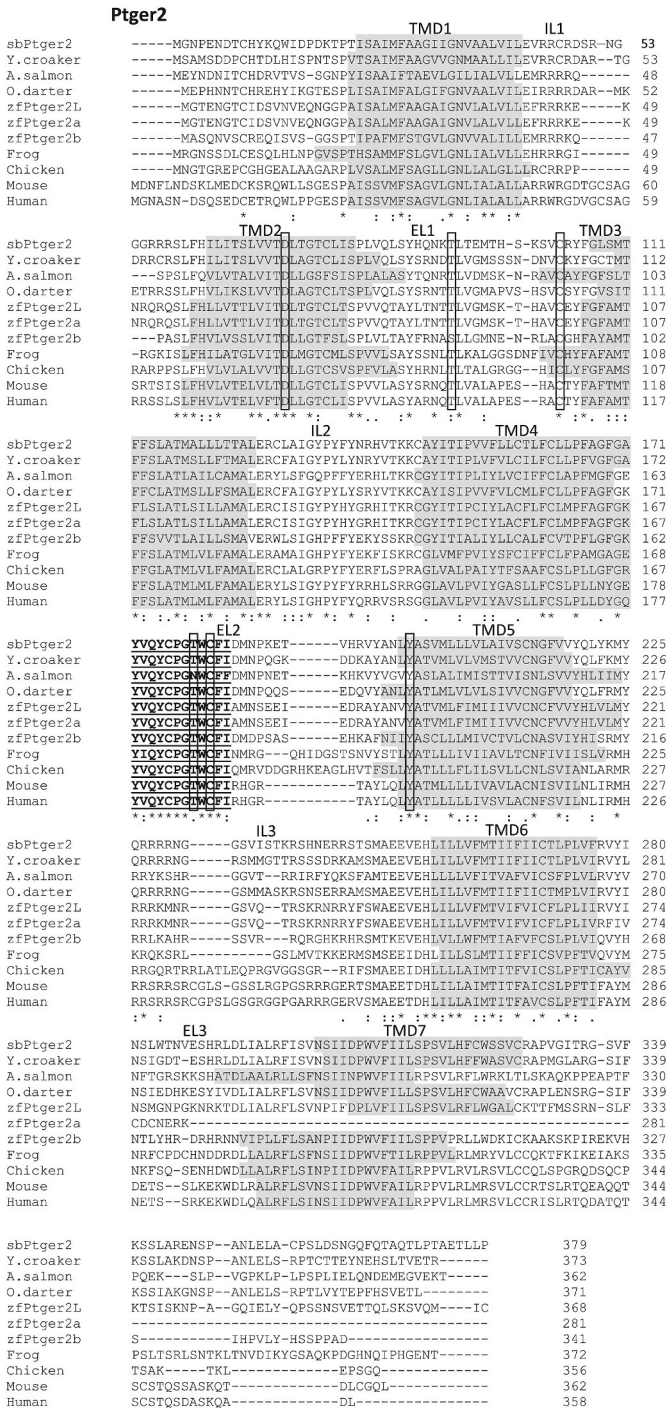

Fig. 4. Sequence alignment and domain organization of seabream Ptger2a sequence with known vertebrate Ptger2 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; () conservative substitutions in one position; (.) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in Table 2. Conserved motifs are in bold and underlined.

Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals. Seabream were killed by cervical dislocation using approved procedures following anaesthetization with clove oil. All efforts were made to minimize animal stress and to ensure that termination procedures were performed efficiently.

2.2. In vivo experimental infections and sampling

Five fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing a sublethal dose (10^8) of exponentially growing *Vibrio anguillarum* R82 cells (Chaves-Pozo et al., 2004). Head kidney, spleen, thymus, liver, gills, blood and peritoneal exudates cells were obtained 4 h after bacterial challenge, a time-point previously described as sufficient to trigger the mobilization of immune cells(Chaves-Pozo et al., 2005). All samples were processed for subsequent real-time RT-PCR (see below).

2.3. Isolation of phagocytes

AGs were obtained by Magnetic-Activated Cell Sorting (MACS), as described previously (Roca et al., 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7) (Sepulcre et al., 2002), washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% FCS (Invitrogen) and then incubated with 100–200 μl per 108 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, G7+ (AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al., 2006). Head kidney macrophage monolayers were then obtained by overnight culture of G7-fractions in serum-free medium as described previously (Roca et al., 2006).

2.4. Cell culture and treatments

Phagocytes (AGs and macrophages) were treated at 23 °C with 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) (Pelegrin et al., 2004) and/or PGE_2 (10 μM, Cayman Chemical) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl, supplemented with 0.1% FCS and 100 IU/ml penicillin and 100 μg/ml streptomycin (Biochrom). Stimulation times were 16 h for AGs and 3 h for macrophages. The concentrations used above have been found to be optimal for the *in vitro* activation of seabream phagocytes (Sepulcre et al., 2007; Montero et al., 2016).

2.5. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and the upper phases containing RNA were purified with RNAqueous Micro Kit (Ambion) according to the manufacturer's protocols. Final RNA concentration was measured at 260 nm by means of the nanodrop system (ND-1000), and RNA quality was checked by electrophoresis. RNA was stored at −80 °C until use. Total RNA was treated with amplification grade DNase I (1 unit/μg RNA, Life Technologies). Then, SuperScrip III RNase H- Reverse Transcriptase (Life Technologies) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 μg of total RNA for 50 min at 50 °C, followed by 15 min at 70 °C. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reactions were performed in 10 μl mixtures, containing 5 μl of 2 × SYBR Green PCR Core Reagents (Applied Biosystems), 0.15 μl of each primer (0.15 μM, final concentration), and 2.5 μl of cDNA. The amplification profile was: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and, finally, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. The RNA was quantified using ribosomal protein S18 (*rps18*) transcript content as housekeeping gene using the comparative Ct method ($2^{-\Delta\Delta Ct}$ for "fold change" and $2^{-\Delta Ct}$ for constitutive expression). The primers used (Table 1) had an amplification efficiency of 2 and their specificity was confirmed by melting curve analysis and the sequencing of each amplified product (Genetic Analyzer ABI PRISM 3130, Applied Biosystems) (data not shown). In all cases, each PCR was performed with

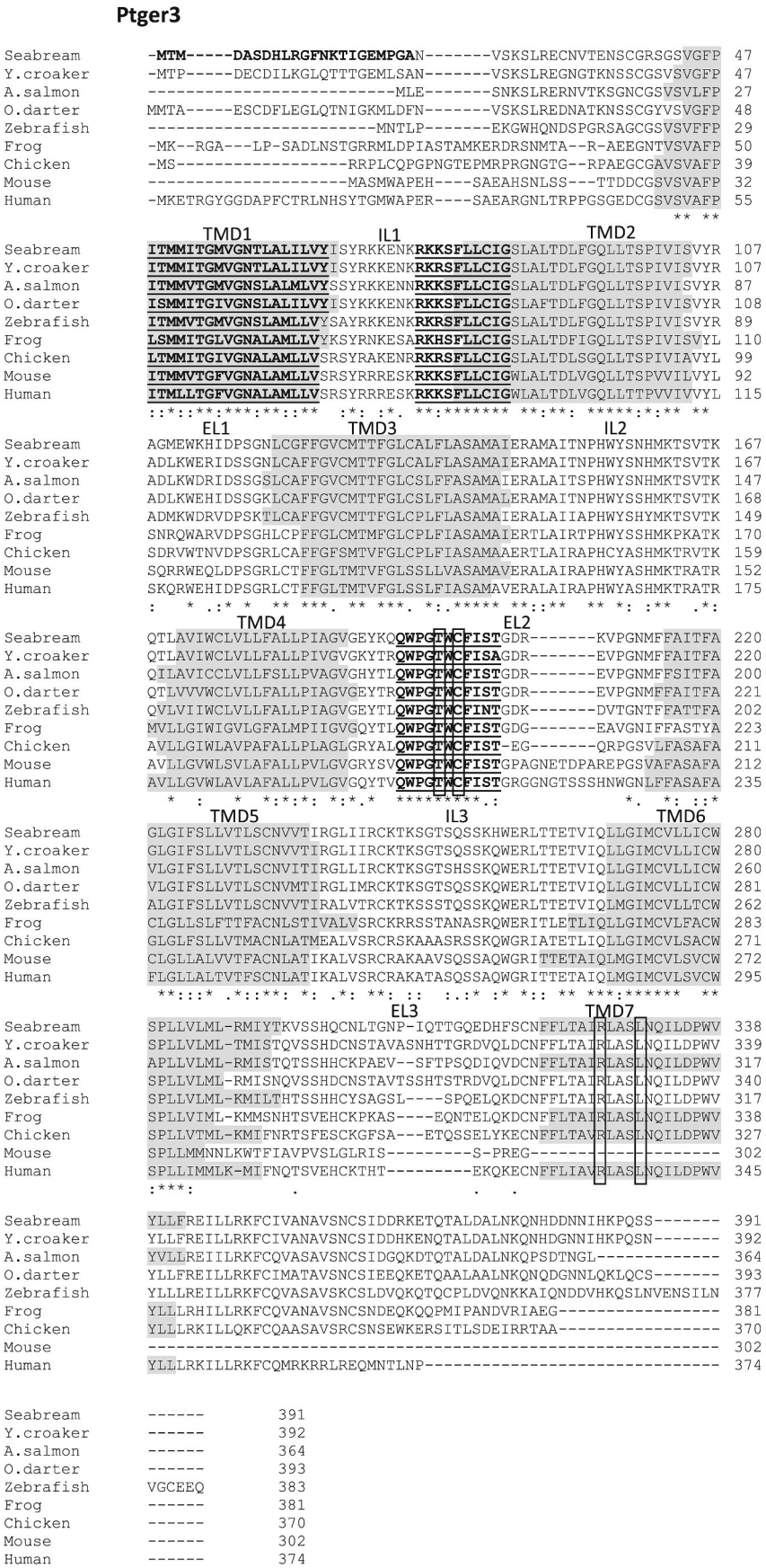

Fig. 5. Sequence alignment and domain organization of seabream Ptger3 sequence with known vertebrate Ptger3 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; () conservative substitutions in one position; (.) semiconservative substitutions in one position. The accession numbers for the sequences used are listed in Table 2. Conserved motifs are in bold and underlined.

Table 3
Amino acid sequence identity (%) between seabream Ptger1-4 and other vertebrates Ptger subtypes.

	Ptger1	Ptger2a	Ptger3	Ptger4
Human	42.8	46.7	61.3	56.8
Mouse	43.8	47.1	60.4	56.2
Chicken	–	44.3	63	58.6
Frog	46.1	45.5	65	64.9
Zebrafish	zEP1a: 67.8 zEP1b: 63.8 zEP1c: 47	zEP2a: 59.8 zEP2L: 58 zEP2b: 49.4	76.1	zEP4a: 66.6 zEP4b: 62 zEP4c: 49
Atlantic salmon	65.8	53.3	79.5	65.2
Yellow Croaker	87.1	74.4	86.2	62
Orange throat darter	88.1	71.3	80.4	84.7

triplicate samples.

2.6. In silico analysis of seabream PGE_2 receptor sequences

Nucleotide sequences of seabream *ptger1, ptger2a, ptger2b, ptger3*and *ptger4* (Accession numbers in Table 2), were obtained from a non-public EST data base. The deduced amino acid sequences were obtained via EMBOSS: Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Areas of seabream protein sequences similar to those of other vertebrate sequences obtained from the NCBI data base were compared after multiple amino acid sequence alignments within the website tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), provided by the European Bioinformatics Institute (EMBL-EBI), in our case: fish (large yellow croaker and zebrafish), reptiles (frog), bird (chicken), mammals (mouse and human). Phylogenetic reconstructions were performed with phylogeny software (http://www.phylogeny.fr/) (Dereeper et al., 2008).) The molecular weight of each protein was determined using the Science Gateway tool (http://www.sciencegateway.org/tools/proteinmw.htm). Finally the structure and domain organization of each receptor were determined using the psipred protein structure predictor, (http://bioinf.cs.ucl.ac.uk/psipred), SMART program, and TMHMM Software (http://www.cbs.dtu.dk/services/TMHMM/) and with the HMMTOP server (http://www.enzim.hu/hmmtop/).

2.7. Cloning the full-length cDNA of seabream Ptger1-4

Sequences of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 were obtained by PCR amplification with Pfu DNA polymerase (Fermentas)using cDNA from seabream head kidney as template. The specific primers used for amplification (listed in Table 1) were designed by using the Primer Designing Tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCRs were performed at low melting temperature: first 5 cycles at 58 °C followed by 30 cycles at 72 °C. The PCR amplified fragments were cloned between *Bam*HI and *Not*I restriction sites into the plasmid pcDNA6/V5-His C (Life Technologies) in frame with the sequence coding for the V5 epitope. All constructs were sequenced by using ABI PRISM 377 (Applied Biosystems).

2.8. Statistical analysis

Data were analyzed by ANOVA and Tukey's multiple range tests to determine differences between groups. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Identification and characterization of genes encoding Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 in gilthead seabream

Searches within a non-publicly EST database allowed us to identify two genes encoding for gilthead seabream Ptger2 designated as Ptger2a and Ptger2b (ENA accession numbers XM_030410287.1 and XM_030410288.1, respectively) and one for Ptger1, Ptger3 and Ptger4 (ENA accession numbers XM_030412901.1, XM_030434233.1 and XM_030436477.1, respectively). The full-length open reading frame (ORF) of *ptger1* (Fig. 1A), *ptger2a* (Fig. 1B), *ptger2b* (Fig. 1C), *ptger3* (Fig. 1D) and *ptger4* (Fig. 1E) are 1140,1140, 885, 1203 and 1341 base pairs (bp), respectively, which translated into the putative 379, 379, 294, 391 and 446 amino acid polypeptides with estimated molecular weights of 41.34, 42.71, 33.46, 43.46 and 49.54 kDa, respectively. The coding sequences for Ptger2a and Ptger2b showed 73% nucleotide identity and differed in the last 312 nucleotides before the stop codon (Fig. 1B and C). The 5′ and 3′ UTR sequences of *ptger1* had 381 and 855 bp, respectively (Fig. 1A). However, the 5′ and 3′ UTR sequences of *ptger2a* are not available in public databases. The sequences of *ptger2b* 5′ and 3′UTR had 90 and 330 bp (Fig. 1C), while ptger3 had 78 and 1203, respectively (Fig. 1D). By contrast, *ptger4* receptor had the longest UTR sequences: 860 bp for 5′UTR and 1414bp for 3′UTR (Fig. 1E).

The pivotal regulatory elements found in each gene are shown in Fig. 1: i) two putative *ccaat* boxes in *ptger2b* (one in the 5′UTR and one in 3′UTR) and three in *ptger4* (two in the 5′UTR and one in 3′UTR), ii) a polyadenylation signal, an important 3′ end element involved in mRNA maturation, transport and translation in eukaryotes (Sachs, 1990), was found in *ptger1, ptger3* and *ptger4* 3′UTRs. iii) three motifs *attta*, associated with the instability of mammalian (Han et al., 1990) and fish (Roca et al., 2007) cytokine mRNAs, were found in *ptger1* 3′UTR, four in *ptger3* and six in *ptger4* (one located in the 5′UTR and five in 3′UTR). The presence of these motifs, especially the sequences with three or more ATTTA motifs, is concomitant with rapid post transcriptional modulation, splicing, mRNA export, degradation, translation activation or inhibition, and turnover (Akashi et al., 1994; Khabar, 2010; Moore et al., 2011). Iv) a consensus sequence (ttatttat) of mammalian inflammatory mediator mRNAs was found in *ptger3* 3′UTR and v) a cytoplasmic polyadenylation element (ttttttttttt) was identified in *ptger4* 3′UTR.

3.2. Polypeptide sequences and topology analysis of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4

The in silico analysis of the deduced amino acid sequences of *ptger1*, *ptger2a*, *ptger2b*, *ptger3* and *ptger4* and of their domain organization revealed that they are G protein-coupled receptors, as has been documented in higher vertebrates (Narumiya et al., 1999). They consist of integral membrane proteins that possess seven transmembrane domains (TMD): the extracellular N-terminal, the intracellular C-terminal, three extracellular loops (EL) and three intracellular loops (IL) (Fig. 2A). However, Ptger2b possesses six instead of seven TMD, and hence two EL and a C-terminal on the extracellular side. (Fig. 2B).

In addition, multiple sequence alignments of Ptger1, Ptger2a, Ptger3 and Ptger4 with those of other vertebrates showed long areas of conserved amino acid residues in all the sequences analyzed. The most conserved regions were located within the seven putative transmembrane domains among the identified seabream Ptger1 (Fig. 3), Ptger2a (Fig. 4), Ptger3 (Fig. 5) and Ptger4 (Fig- 6) and their respective orthologs. The identities between whole-length seabream Ptger subtypes and other vertebrates Ptger ranged from 42.8% to 88.1% (Table 3). The motif TWCFI was identified in the second extracellular loop of seabream Ptger1 (Fig. 3), Ptger2a (Fig. 4), Ptger3 (Fig. 5) and Ptger4 (Fig. 6) and in those of the other sequences analyzed with the exception of zebrafish Ptger1c, frog Ptger1 and yellow croaker Ptger4, which contain leucine instead of isoleucine (Figs. 3–6). This motif constitutes part of the 12 highly conserved amino acid residues in Ptger2a, Ptger2b (YVQYCPGTWCFI) and in Ptger3 (QWPGTWCFISTG) of most of the vertebrates mentioned in this work, except chicken Ptger3, which lacks the G (Figs. 2C and 4). These invariant residues form part of the second extracellular loop in all the mentioned vertebrate sequences, and has been suggested to act not only as a link between the transmembrane domains, but also to have a conserved structural role (allosteric

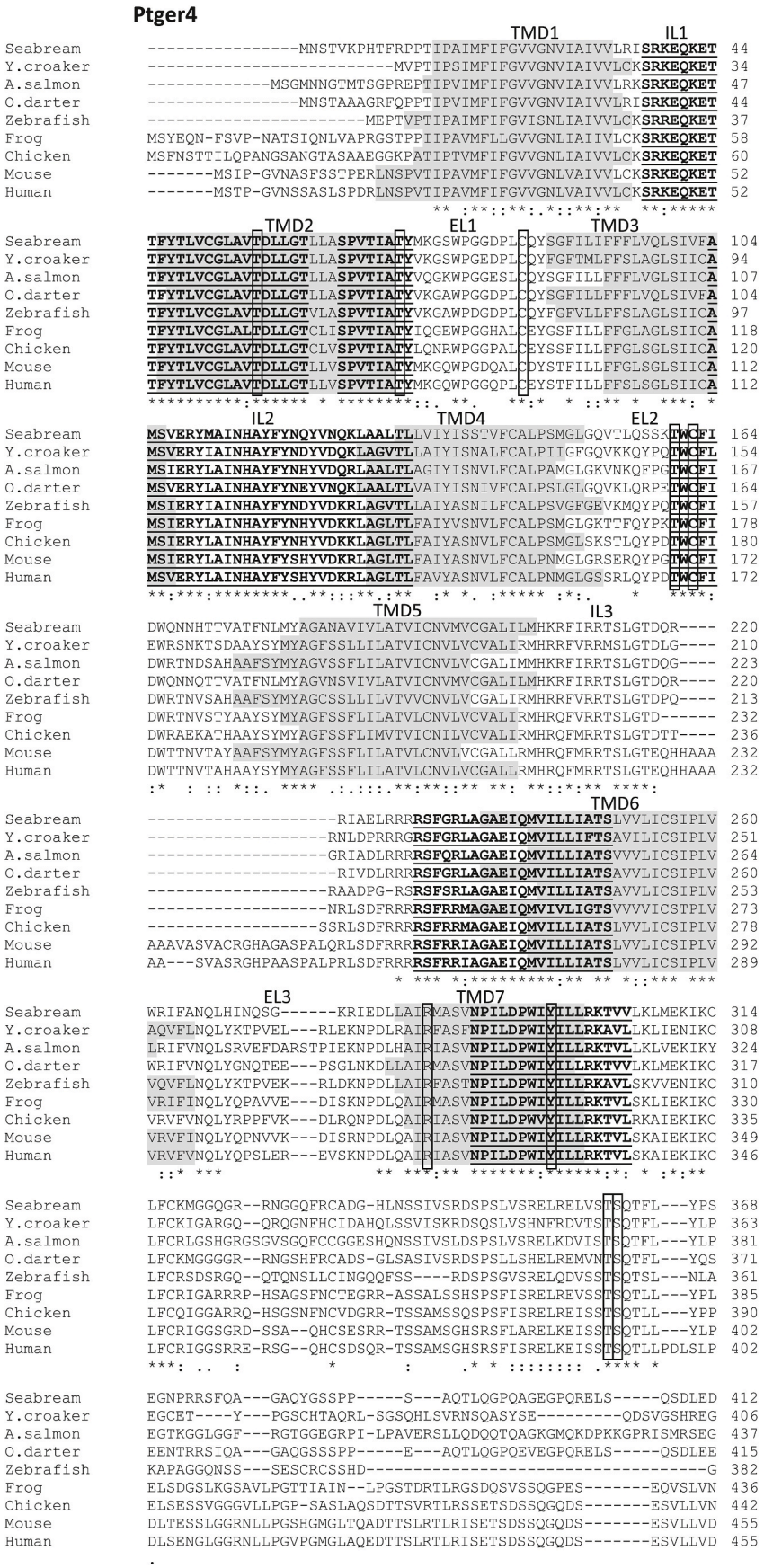

Fig. 6. Sequence alignment and domain organization of seabream Ptger4 sequence with known vertebrate Ptger4 sequences. Transmembrane domains (TMD) are shaded, extracellular loop (EL), intracellular loop (IL). (*) Identity in one position; () conservative substitutions in one position; (.) semiconservative

substitutions in one position. The accession numbers for the sequences used are listed in Table 2. Conserved motifs are in bold and underlined. Signal peptides are in bold.

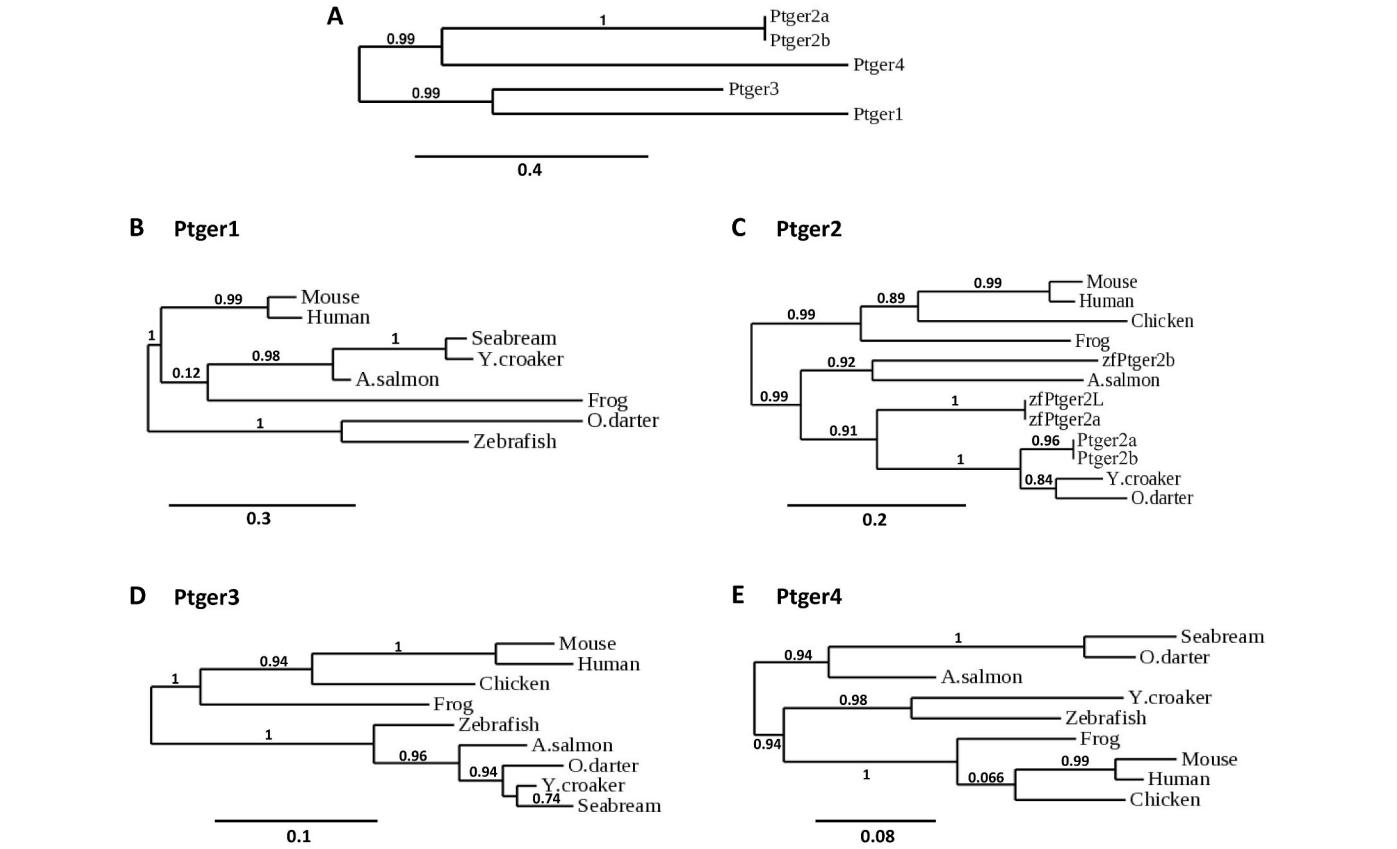

Fig. 7. Phylogenetic relationships seabream Ptger subtypes sequences. Phylogenetic tree of seabream Ptger1-4 (A) and vertebrate Ptger subtypes and seabream Ptger1 (B), Ptger2a and Ptger2b (C), Ptger3 (D) and Ptger4 (E) polypeptides. The tree was generated by phylogeny.fr software (Dereeper et al., 2008) using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances, while the vertical lines have no significance. ENA accession numbers are listed in Table 2.

conformation) and functional actions related with GTP sensitivity as well as agonist binding interaction (Stillman et al., 1998). In this context, it is important to mention that the complex G protein/GTP plays an important role in the receptor's passage from inactive "R" to the active conformation "R*" in GPCR (De Lean et al., 1980; Nataraj et al., 2001; Samama et al., 1993; Stillman et al., 1999; Venkatakrishnan et al., 2013). Furthermore, seabream Ptger4 sequence has more highly conserved regions in their TMD, EL and IL than Ptger1, Ptger2a and Ptger3 (Fig. 6). Notably, key residues responsible for structural as well as functional aspects of mammalian Ptger, were also found in seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4. In particular, the conserved T (in EL1) (Stillman et al., 1999), D (TMD2), C (EL1 and 2) (Savarese and Fraser, 1992) and Y (TMD5) (Suganami et al., 2016), as well as the R (TMD7, a charged amino acid residue that is further conserved in the TMD7 of all PGE_2 receptors of the vertebrates mentioned in the present study except in zebrafish Ptger2b. In support of this finding, it was suggested previously that this arginine is one of the residues that may be involved in relevant functional aspects of these receptors, including conformation stability, ligand binding and signal transduction in zebrafish (Kwok et al., 2012) and in human (Audoly and Breyer, 1997; Neuschäfer-Rube et al., 2004; Wilson et al., 2001). The T and S residues of the C terminus of Ptger4, responsible for agonist-induced short-term desensitization (Bastepe and Ashby, 1999), were also found in seabream Ptger4 (Fig. 6).

3.3. Phylogenetic analysis of seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4

The phylogenetic tree constructed with the Neighbor-Joining method, based on full amino acid sequences of seabream Ptger1-4, revealed two branches. The fist branch comprised Ptger2 and Ptger4 and the second branch comprised Ptger1 and Ptger3 (Fig. 7A). Furthermore, the phylogenetic tree constructed with mammalian and teleost Ptger sequences formed two clusters (Fig. 7B–E). Moreover, gilthead seabream Ptger1-4 have the closest phylogenetic relationship with their respective receptors of other fish species belonging to the Perciformes order, followed by Salmoniformes (Fig. 7B–E).

3.4. Gilthead seabream Ptger1-4 are differentially distributed and modulated upon bacterial infection

To gain further insight into the role of the Ptger1-4 immune response in gilthead seabream, RT-qPCR was used to analyze the expression pattern of their corresponding genes as well as their modulation upon bacterial challenge. Seabream *ptger1*, *ptger2a*, *ptger3* and *ptger4* were expressed in all the tissues examined, including such key immune organs as head kidney (HK), spleen, thymus and gills (Fig. 8). In particular, the highest mRNA levels of *ptger1* were found in spleen, followed by HK, thymus, gill and peritoneal exudate (PE, the site of bacterial injection) (Fig. 8B). In a similar way, the most abundant transcript levels of *ptger3* were also found in spleen and gill, followed by thymus, HK, and liver

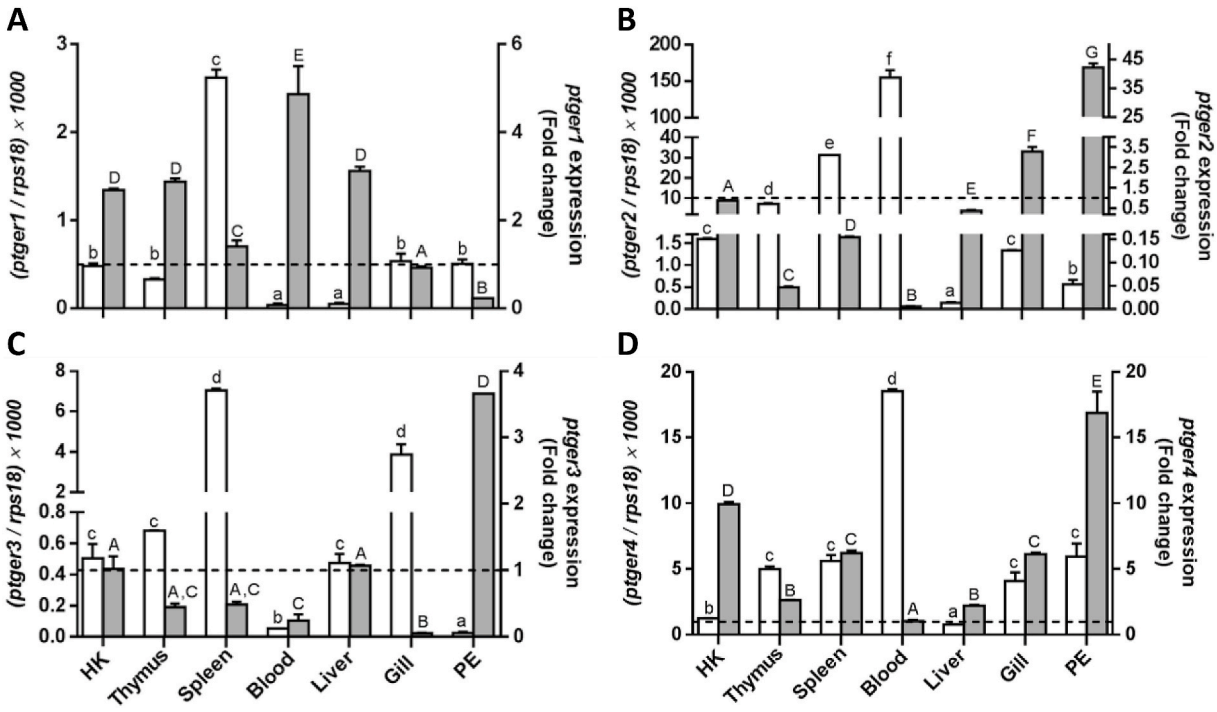

Fig. 8. Tissue expression pattern of seabream Ptger subtypes. Basal mRNA levels (left y axis, white bars) and those following bacterial challenge (right y axis, gray bars) of *ptger1* (A), *ptger2a* (B), *ptger3* (C) and *ptger4* (D) were determined by real-time RT-PCR in the indicated tissues of control adult specimens or following a 4 h challenge with 10^8 cells of *Vibrio anguillarum*. The results are representative of five fishes. The constitutive expression is shown relative to *rps18* and the regulation of the expression following infection is shown as the mean ± S.E. of the mRNA fold change in infected fish relative to control fish (indicated with horizontal dashed lines). Different letters denote statistically significant differences among the groups according to a Tukey test ($p < 0.05$). The groups marked "A" did not show statistically significant differences from non-infected fish.

(Fig. 8C). Interestingly, the weakest mRNA levels of *ptger1* and *ptger3* were found in blood (Fig. 8C). By contrast, the highest mRNA levels of *ptger2a* and *ptger4* were found in blood (Fig. 8B–D), followed by spleen and thymus for *ptger2a* (Fig. 8B) and by spleen, thymus, PE and gill for *ptger4* (Fig. 8D). However, it is important to note that the magnitude order of the mRNA levels of *ptger2a* is higher compared to those of *ptger4* and much higher compared to *ptger1* and *ptger3* ones (Fig. 8A–D).

Furthermore, infection of fish with the pathogenic bacterium *V. anguillarum* resulted in increased mRNA levels of *ptger1*, mainly in blood followed by HK, thymus and liver (Fig. 8A). However, bacterial challenge led to decreased mRNA levels of *ptger1* in PE (Fig. 8A). By contrast, the mRNA levels of *ptger3* did not significantly change, or slightly decreased, in all the tissues examined, with the exception of PE, in which increased four-fold. Similarly, bacterial challenge resulted in increased mRNA levels of *ptger2a* and *ptger4*, mainly in PE (Figs. 8B–4D) followed by gill in the case of *ptger2a* (Fig. 8B), and HK, spleen and gill in the case of *ptger4* (Fig. 8D). However, the mRNA levels of *ptger2a* did not significantly change in HK, and even decreased dramatically in blood (the highest basal expression site), spleen and thymus after bacterial challenge (Fig. 8B). However, the mRNA levels of *ptger4* increased in all organ tissues analyzed, mainly in PE, but not in blood, where they did not change (Fig. 8D). Taken together, these results showed a different distribution and modulation pattern of the four PGE_2 receptors in the main immune organs of gilthead seabream.

3.5. Expression of gilthead seabream Ptger1-4 in professional phagocytes

We next analyzed the constitutive expression of *ptger1*, *ptger2a*, *ptger3* and *ptger4* in seabream professional phagocytes, macrophages and AGs, as well as the effect of their activation with VaDNA or/and PGE2 (the receptor ligand) in the mRNA levels of those receptors. It is important to note that although the methods used for phagocyte isolation could induce some level of activation, previous studies showed that this induction was minimal compared to those of VaDNA for other activation markers, like il1b and tlr5 (Sepulcre et al., 2007) or pges (Montero et al., 2016). The results showed that the four receptors were constitutively expressed in both cell types, more strongly in macrophages than in AGs, with the exception of *ptger1* (Fig. 9A). Interestingly, while AGs showed similar mRNA levels of the four receptors, the transcription levels of the genes encoding for Ptger2a and Ptger3 in macrophages were much higher than those of Ptger1 or Ptger4 (Fig. 9A). Furthermore, the results revealed that, in macrophages, the mRNA levels of *ptger1*, *ptger2a*, *ptger3* and *ptger4* decreased upon stimulation with VaDNA (Fig. 9B–E). However, treatment of macrophages with PGE_2, resulted in a similar expression pattern of *ptger1* and *ptger3*, whose mRNA level decreased (Fig. 9B and D), and of *ptger2a* and *ptger4*, whose mRNA levels did not significantly change (Fig. 9C and E). In addition, the joint treatment of macrophages with PGE_2 and VaDNA had a synergistic effect in the reduction of the mRNA levels of *ptger1*, but led to a less pronounced decrease in the transcription levels of *ptger3*. Furthermore, the joint treatment of macrophages with PGE2 and VaDNA abolished the effect that VaDNA alone had on the reduction of *ptger2a* and *ptger4* transcript levels.

In the case of AGs, stimulation by VaDNA or PGE2 resulted in increased mRNA levels of *ptger2a* and *ptger4*, especially in the case of PGE_2 (Fig. 9C and E). In addition, treatment of AGs with PGE2 and VaDNA together abolished the effect of PGE2 in the induction of *ptger2a* and *ptger4* transcript levels (Fig. 9C and E). In the case of *ptger1* mRNA levels, the individual treatment of AGs with VaDNA or PGE_2 had a much weaker effect, and their combined use resulted in a dramatic decrease (Fig. 9B). However, the results differed in the case of *ptger3*, when VaDNA increased *ptger3* mRNA levels and PGE_2 had no effect on its own. However, the combination of both stimuli resulted in the lower induction of *ptger3* transcript levels compared with VaDNA alone (Fig. 9D). All these results further demonstrate that seabream phagocytic cells are armed with Ptger1, Ptger2a, Ptger3 and Ptger4, which allows them to

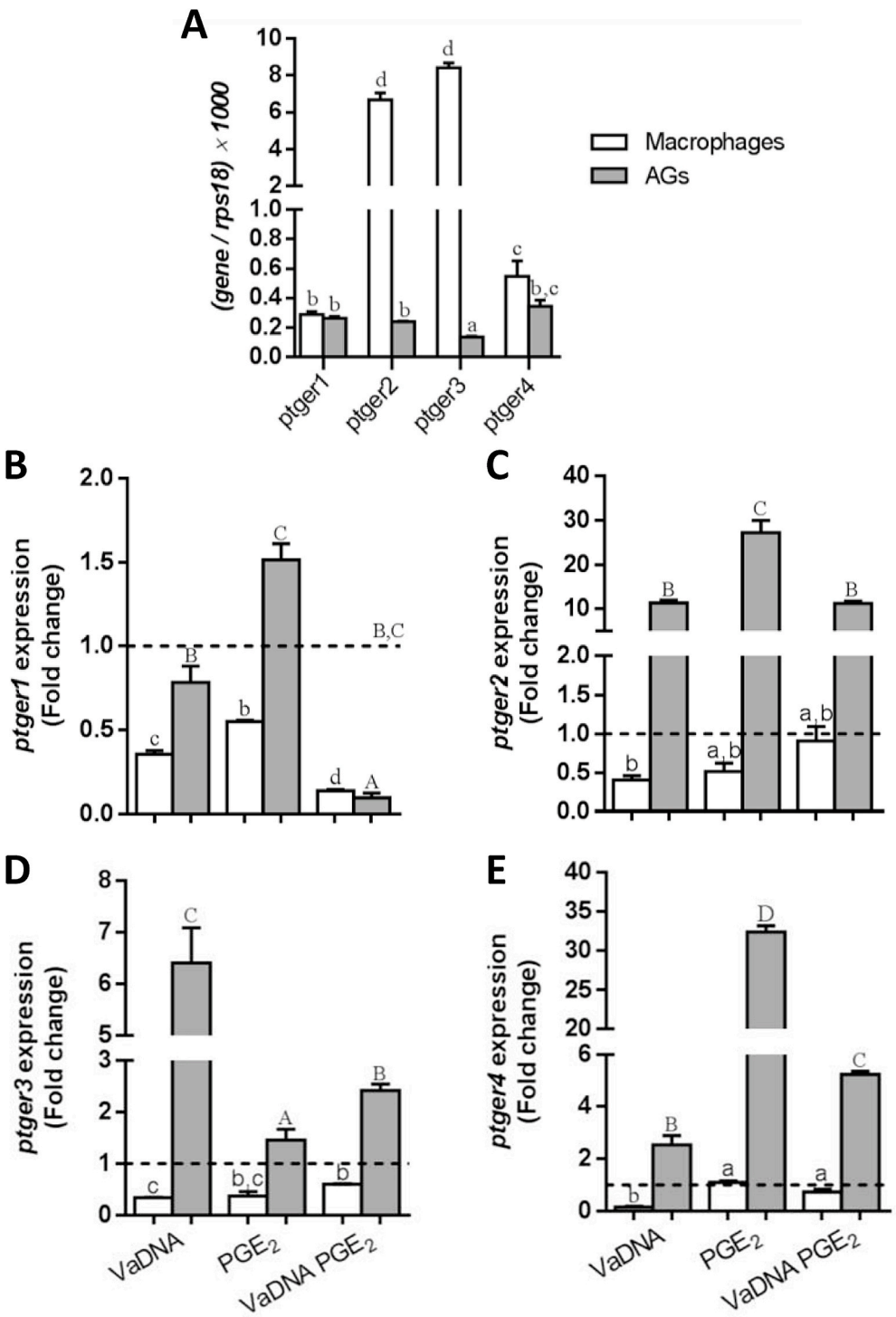

Fig. 9. Expression pattern of seabream Ptger subtypes in professional phagocytes. Basal mRNA levels of seabream Ptger subtypes in macrophages (white bars) and GAs (gray bars) (A) and following stimulation with VaDNA and/or PGE_2. Levels of *ptger1* (B), *ptger2a* (C), *ptger3* (D) and *ptger4* (E) were determined by real-time RT-PCR. The results are representative of six independent experiments The constitutive expression is shown as relative to *rps18* and the regulation of expression following stimulation is shown as the mean ± S.E. of the mRNA fold change in non-stimulated cells. Different letters denote statistically significant differences among the groups. The groups marked "a" for macrophages or "A" for AGs did not show statistically significant differences from non-stimulated cells with the exception of Ptger1 where this group is market "B,C".

respond to their ligand, PGE_2, which interfere in the regulation of the mRNA levels of each receptor by PAMPs. Furthermore, in the light of our data, it is tempting to speculate that each phagocyte population would play different roles in the innate immune responses through these receptors, however further functional studies are required.

4. Discussion

Recent studies from our group have shown that PGs, including PGE_2, play a key role in the inflammatory process in the most divergent group of vertebrates, teleost fish (Gómez-Abellán et al., 2015; Gómez-Abellán and Sepulcre, 2016; Montero et al., 2016; Tyrkalska et al., 2016). However, studies concerning the PGE_2 signaling pathways are scarce. In the present study, seabream PGE_2 receptors, Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4, were cloned and characterized. Multiple alignment, structure domain and phylogenetic tree analysis revealed that the characterized receptors are the bona fide orthologs of mammalian PTGERs, with the typical domain organization of G protein-coupled receptors. Furthermore, a variant of seabream Ptger2a, called Ptger2ab, was also identified and found to be structurally composed of six TMD rather than the seven present in all PGE_2 receptor subtypes of the vertebrates mentioned in this study, similar to zebrafish Ptger2a and

mouse Ptger3, which have six TMD. It is possible that these two isoforms exhibit different degrees of sensitivity with regard to PGE_2 binding, resulting in different levels of action. Two isoforms, Ptger2a and Ptger2b, have been identified in zebrafish, in which both enhance cAMP synthesis (Tsuge et al., 2013). Interestingly, putative ccaat boxes and polyadenylation signals were found in the UTRs of ptger2a and ptger4, as they have been in higher vertebrates (Arakawa et al., 1996; Foord et al., 1996; Locker and Buzard, 1990; Smock et al., 1999). Furthermore, motifs associated with mRNA instability were identified in *ptger1*, *ptger3* and *ptger4*; however, cytoplasmic polyadenylation elements were identified only in *ptger4* UTRs. In addition, seabream Ptger1, Ptger2a, Ptger2b, Ptger3 and Ptger4 share with their vertebrate orthologs the key residues responsible for structural as well as functional aspects, namely the conserved threonine (Stillman et al., 1999), aspartic acid, cysteine (Savarese and Fraser, 1992) and tyrosine (Suganami et al., 2016) residues. Conserved arginine of the seventh TMD (Narumiya et al., 1999), which is preserved among vertebrates (Yokoyama et al., 2013) and targeted by N/O glycosylation, as well as the threonine and the serine residues of the C terminus responsible for agonist-induced short-term desensitization (Bastepe and Ashby, 1999) were only found in Ptger4. The motifs of the extracellular and intracellular loops involved in G protein coupling and/or ligand binding are conserved among fish and their orthologs (Kwok et al., 2012), which was further confirmed by the phylogenetic analysis which showed that seabream PGE_2 receptors clustered into two groups: one involving higher vertebrates and the other encompassing all fish species. These new findings, together with previous results (Margan et al., 2012; Stillman et al., 1999; Hagiwara et al., 2014; Guo et al., 2015), support the idea that PGE_2 signaling through Ptger1-4 subtypes predated the split of fish and tetrapods more than 450 million years ago.

Several reports exist about the effect of PGs on immune-related activities in fish, most of them focused on the biological activity of PGE_2 (Gómez-Abellán and Sepulcre, 2016). The variety of effects that PGE_2 can elicit, may reflect the presence of specific Ptger subtypes in different tissues and cell types. We have shown herein that gilthead seabream Ptger subtypes were expressed in all the immune tissues analyzed, including HK, spleen, blood, gill, thymus and PE, but with different distributions. In this respect, the results pointed to two distinct expression patterns: one for *ptger1* and *ptger3* and another for *ptger2* and *ptger4*. The highest mRNA levels of the genes coding for Ptger1 and Ptger3 were found in spleen, while those coding for Ptger2a and Ptger4 were also observed in blood. However, *ptger2a* and *ptger4* were the most abundant subtypes in most of the tissues analyzed, unlike in mouse, where *Ptger3* and *Ptger4* are the most robustly distributed. It is important to note that the basal expression profile of the four receptors differs in the main phagocytic cell types of seabream, being *ptger2a* and *ptger3* more strongly expressed in macrophages than in AGs and showing AGs similar basal mRNA levels of *ptger1-4*. Hence, the basal expression profile of *ptger1*-4 in the different organs reflects the contribution of the breadth of cell types present in the specific organ, not only of macrophages and AGs.

Furthermore, seabream challenged with *V. anguillarum* modulated the expression of genes encoding the different Ptger subtypes, *ptger2a* and *ptger4*, showed similar expression profiles, PE (the site of bacterial injection) being the tissue with the highest induction rate of the mRNA levels of both receptors, and blood with lowest rate. In contrast, *ptger1* showed a modulation profile that was the opposite of that shown by the rest of the *ptger* subtypes. Taken together, these results suggest not only that mRNA levels in the specific tissues are modulated in response to bacterial challenge but also could reflect the mobilization of different immune cells from one organ to another (Chaves-Pozo et al., 2005). To get further into this point, we also analyzed the constitutive expression of the genes encoding Ptger1-4 and their modulation by PAMP and ligand stimulation in the main innate immune cells of seabream. The results showed that stimulation of phagocytic cells with VaDNA and PGE_2, alone or together, up-regulated the mRNA levels of *ptger2a*, *ptger3* and *ptger4* in AGs, but not in macrophages, where the levels decreased very slightly or did not change. This data, together with those previously reported showing that seabream infection with *V.anguillarum* results in AGs recruitment and activation at the injection site at the time tested herein (Chaves-Pozo et al., 2005), support the hypothesis that mobilization of AGs from different organs contribute to the mRNA levels of *ptger1-4* in the specific tissues after bacterial challenge. Furthermore, the results confirmed the *in vivo* results by demonstrating the similar regulation of *ptger2a* and *ptger4* expression by VaDNA or/and PGE_2 in both AGs and macrophages. Nevertheless, *ptger1* and *ptger3* showed a similar degree of modulation in macrophages, but behaved in a totally different way in AGs. This differential regulation of *ptger1-4* in macrophages and AGs is in agreement with the fact that PGE_2 promotes M2 polarization of macrophages but deactivates AGs.

In conclusion, the above results, taken together, suggest a role for G protein-coupled PGE_2 receptors, that is conserved from fish to mammals, thus adding for our understanding of their roles in the modulation of the teleost immune response.

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